A simple, surface antigen-specific, monoclonal antibody based dot-ELISA for the identification of *Listeria monocytogenes*

Srividya Y, Joseph Kingston J, H. S. Murali, H.V. Batra

Abstract- *Listeria monocytogenes* is a human pathogen, transmission of this bacterium is mainly through food especially milk and milk products, and also from non vegetarian food products like mutton, poultry, beef, fish and pork. *L. monocytogenes* outbreaks are due to contaminated foods which emphasizes the need to develop a rapid and cost effective detection.

Monoclonal antibodies with specificities of different epitopes were generated against whole cell antigens of formalinized *L. monocytogenes* cells. Six MAbs from the fusion were tested against 12 *L. monocytogenes* standard strains and 69 wild-type isolates obtained from naturally contaminated food products, Mab107 showed specificity to *L. monocytogenes* in Plate ELISA and Western blot. Utilising this clone a simple monoclonal antibody based dot-ELISA system was standardized for rapid detection of *L. monocytogenes* from contaminated food stuffs. Developed system was successfully evaluated on to different food matrices collected from local markets of Karnataka and ELISA results were cross checked with conventional method and PCR method, where, listeriolysin O gene was targeted and standardised in house. To assess the cross-reactivity of the Mab, tests were made using standard strains of *Listeria* along with that closer group of other *Listeria* species and other bacterial genera. The cost effectiveness, sensitivity, specificity and reliability of the method proved to be good. Therefore, the system can be used in routine diagnostic laboratories.

Index Terms- *Listeria monocytogenes*, Monoclonal antibodies, PALCAM agar, PCR.

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1 INTRODUCTION

The genus *Listeria* is comprised of 6 species (*L. monocytogenes, L. ivannovi, L. innocua, L. welshimeri, L. seeligeri, L. grayi*), of which *L. monocytogenes* is a human pathogen where as *L. ivannovi* is an animal pathogen. All *Listeria* species are morphologically identical they are short (0.5 × 1 - 2 micrometers), Gram-positive, catalase-positive, nonacid-fast, nonsporulating, facultative anaerobic rods. They exhibit a characteristic tumbling motility at 20 °C to 25 °C [1], [2], [3]. Transmission of *L. monocytogenes* is mainly thorough food especially milk and milk products, and also non vegetarian food products like mutton, poultry, beef, fish and pork [2]. Listeriosis is relatively rare disease and occurs primarily in pregnant woman, newborn infants, elderly patients, and patients who are immunocompromised [4]. Since *L. monocytogenes* is present in slaughter animals and subsequently in raw meat and poultry as well as other ingredients, it can be continuously introduced into the processing environment. Other characteristics of *L. monocytogenes* that makes it a formidable pathogen to control are its heat and salt tolerance and its ability to grow at refrigeration temperatures and survive at freezing temperatures. Two types of Listeriosis have been reported; Invasive listeriosis and non-invasive listeriosis. Invasive Listeriosis results in adverse outcome such as septicemia, meningitis, encephalitis, abortion or stillbirth, materno-fetal infection, endocarditis, cutaneous infections and though rare, it may cause focal infections, such as endophthalmitis, septic arthritis, osteomyelitis and pleural infection Non-invasive listeriosis causes gastrointestinal illness, which may result in chills, diarrhea, headache, abdominal pain and cramps, nausea, vomiting, fatigue, and myalgia [4]. Listeriosis has the high case fatality rate (20-30% of cases), the long incubation time and the predilection for immunocompromised [1], thus emerging as an atypical foodborne illness of major public health concern.

The Centers for Disease Control and Prevention (CDC) has estimated that approximately 2,500 cases of listeriosis occur annually in the United States [5], [6]. Until 1973 there was no case report of listeriosis in India, Bhujwala et al. studied in the same year and reported 3 cases, in 1981 Bhujwala *et al*. identified 9 cases out of 670 women with bad obstetric history [7].

A case of listeriosis with a poor fetal outcome in second trimester of pregnancy was reported by Varsha *et al* in 2003. *L. monocytogenes* outbreaks due to contaminated foods emphasize the need to develop a rapid detection [8], [9], [10]. Several culture and biochemical methods have been developed and used successfully torecover and confirm *L. monocytogenes* [11], [12].

Conventional culture based methods for identification of *L. monocytogenes* is tedious and time consuming (ISO 11 290-1:1996a, b; ISO 10 560-1:1999), and these methods can require 5–10 days to complete [13], [14], [15]. In recent years attempts have been made to explore new methods based on monoclonal antibodies to identify the *L. monocytogenes* form food and other contaminated samples [16], [17]. DNA/RNA probes and DNA amplification with polymerase reaction [18], [19], [20] for detection of *L. monocytogenes* are very promising. Enzyme immune assays based on several monoclonal antibodies have been developed in an effort to detect *L. monocytogenes* in food and clinical samples [21], [22], [23], [15].

Thus, there is still a lacuna in reliable, rapid and cost effective detection system based on monoclonal antibodies to detect *L. monocytogenes* from contaminated food samples. Considering those facts, in present study we developed a simple antibody based dot-ELISA system for rapid detection of *Listeria* from contaminated food stuffs. Developed system was successfully evaluated on to different food matrices collected from local markets of Karnataka

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and ELISA results were cross checked with conventional and PCR method as well.

2 MATERIALS AND METHODS

2.1 REAGENTS AND CHEMICALS

Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), streptomycin, penicillin, fetal bovine serum (FBS), polyethylene glycol 2000 (PEG 2000), 3,3,5,5-tetramethylbenzidine (TMB), Diaminobenzedene (DAB) and myeloma cells (Sp2/0-Ag14) were purchased from Sigma Aldrich (St Louis, MO, USA). RPMI1640 culture medium was obtained from Gibco (Carlsbad, CA, USA). Goat anti-mouse IgG horseradish peroxidase (HRP) was obtained from sigma (Sigma, USA). The working buffers used included 0.1 M PBS, pH 7.4 (0.1 M phosphate buffer, 0.138 M NaCl, 0.0027 M KCl), and PBS with Tween 20 (PBST; 0.01M PBS, 0.05% Tween 20). IsoStrip mouse monoclonal antibody isotyping kit was obtained from Sigma Aldrich (Sigma Aldrich, USA). Unless otherwise mentioned, all analytical grade reagents were obtained from Merck, Mumbai, India. Eight-week-old BALB/c mice were obtained from the animal facility, Defence Research and Development Establishment, India. ELISA reader (Bio- Rad Model 550 Microplate Reader (Bio-Rad, Hercules, CA, USA)) was used.

2.2 BACTERIAL STRAINS AND CULTURE CONDITIONS

2.2.a. Standard strains: The bacterial strains used in the study are listed in table 1. Reference strains were obtained from American Type Culture collection (Hi-Media, Mumbai).
2.2.b. Isolates: The isolates used in this study were recovered from mutton, poultry, fish, pork samples collected from various food agencies located in Karnataka state. The samples were inoculated into UVM broth overnight and strains were isolated using PALCAM agar with selective supplement. Pure cultures were maintained on BHI broth overlayerd with 15% glycerol at -80 ^{o}C.
2.2.c. Other bacterial strains: *E. coli* ATCC 9637, *S. typhimurium* ATCC 1408, *Staphylococcus aureus* ATCC 2124, *Bacillus cereus* ATCC 1457, *Bacillus subtilis, Enterobacter aerogens, Aermonas hydrophila, Vibrio parahemolyticus* ATCC 17802, *Shigella boydii* MTCC 1457 cells were grown on BHI broth and maintained in 4 ^{o}C for further use (Table 1).

Table 1
Bacterial strains used in the study

Sl.no	Bacterial strains	Source
1	*L. monocytogenes ATCC 13593*	Himedia
2	*L. monocytogenes ATCC 15313*	Himedia
3	*L. monocytogenes ATCC 19115*	Himedia
4	*L. monocytogenes ATCC 19114*	Himedia
5	*L. monocytogenes ATCC 7644*	Himedia
6	*L. monocytogenes NCIM 839*	Himedia
7	*L. monocytogenes NCIM 1143*	Himedia
8	*L. monocytogenes ATCC 19111*	Himedia
9	*L. monocytogenes ATCC 19112*	Himedia
10	*L. monocytogenes ATCC BAA 751*	Himedia
11	*L. innocua ATCC 33090*	Himedia
12	*L. ivannovi ATCC BAA 139*	Himedia
13	*L. ivannovi ATCC 19119*	Himedia
14	*L. welshimeri ATCC 35897*	Himedia
15	*L. seeligeri ATCC 35967*	Himedia
16	*L. grayi ATCC 25401*	Himedia
17	*L. grayi ATCC 700545*	Himedia
18	*Staphylococcus aureus ATCC 600799*	Himedia
19	*Staphylococcus aureus NCIM 2120*	MTCC
20	*Bacillus cereus ATCC 14579*	Himedia
21	*Bacillus thurengenesis ATCC 16872*	Himedia
22	*Staphylococcus aureus ATCC 2124*	Himedia
23	*Staphylococcus aureus ATCC 2120*	Himedia
24	*Salmonella typhimurium ATCC14028*	Himedia
25	*Shigella boydii MTCC 1457*	Himedia
26	*Escherichia coli ATCC 9637*	Himedia
27	*Vibrio parahemolyticus ATCC 17802*	Himedia
28	*Aeromonas hydrophila*	Himedia

2.3 GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST *Listeria monocytogenes*

2.3.a. Preparation of formalinized cells for immunization:
Overnight cultures of *Listeria monocytogenes* ATCC 19115 were harvested and washed with sterile PBS. Cells were resuspended in commercial neutral-buffered formalin (0.4%,v/v) in 1X PBS then, incubated overnight at room temperature. After incubation cells were pelleted and dissolved in PBS buffer. The cell density was measured and concentration was made upto 1.0×10^9 CFU/ml by Mcfarland standards. Formalin-killed *L. monocytogenes* cells were stored at -20 ^{o}C until use.

2.3.b. Immunization of animals
Two female BALB/c mice, 8-10 weeks of age, weighing approximately 18g, were injected at 0, 8, 16, 24, 32 days subcutaneously with 10^9 cells in 200 µl of sterilized PBS. The mixture was emulsified with an equal volume of Freund's complete adjuvant for first injection and the further injections were given with Freund's incomplete adjuvant. Three days after 5th injection serum was collected from the retro-orbital plexus of both mice [24] and titres of antibodies were determined by an indirect ELISA. Once high titre is reached intraperitoneal booster injection without adjuvant was given 3 days before spleen cells are extracted for fusion from hyperimmune mice.

2.3.c. Generation of monoclonal antibodies (Hybridoma technology)
Hybridoma was carried as per [25] with minor modifications, the spleens of the immunized mice were aseptically removed and fused with Sp2/0-Ag14 cells. Sp2/0-Ag14 cells were cultured in RPMI1640 with 100 mg/ mL streptomycin and 100 U/mL penicillin, in a CO_2 incubator set at 37 ^{o}C and 5% CO_2 concentration. Mouse lymphocytes (10^8 cells) were mixed with the Sp2/0-Ag14 myeloma cells at the ratio of 5-10:1 and centrifuged at 2000 rpm for 5 min. One milliliter volume of PEG 2000 was warmed to 37 ^{o}C and dropped onto the cells pellet within 1 min. The fused cells were centrifuged for 10 min and resuspended in 20 mL complete medium with HAT. Cells were seeded into 96-well culture plates with feeder layers of mouse peritoneal macrophages, and extended in a CO_2 incubator set at 37 ^{o}C and 5% CO_2 concentration. After 5 days, the antibody-secreting hybridoma cells were detected by indirect ELISA. Positive hybridoma cells were grown in hypoxanthine and thymidine (HT) medium. The hybridoma cells that produced the most sensitive supernatant fluid were cloned using the limited dilution and extension method. Sub-cloning was repeated three times to obtain positive mAb producing cells, then amplified and frozen in liquid nitrogen.

2.4. CHARECTERIZATION OF ANTIBODIES

2.4.a. Indirect ELISA

Indirect ELISA was performed to determine the sensitivity and specificity of mAB's. about 0.2 µg of whole cell lysate (sonicated antigen) of *L. monocytogenes* ATCC 19115 was coated with carbonate bicarbonate buffer (pH 9.6) onto 96-well microtiter plate and incubated overnight at 4 ^{o}C the plates were then blocked with 2 % BSA with 1X PBS and incubated overnight at 4 ^{o}C, for minimising nonspecific binding by blocking unoccupied solid-phase sites and 50 µl of culture supernatants of hybridomas which has to be tested was added (50 µl), incubated at room temperature for 1 h then, 50 µl of anti-mouse IgG conjugate (1:1000) was added to each well and incubated for 1 h at room temperature. After each step of incubation washing was done with PBST (1X PBS + 0.05 % Tween) to remove unbound antibodies, the colour was developed using O-phenylenediamine dihydrochloride (Sigma, USA) and H_2O_2 in 0.15 M citrate phosphate buffer (pH 5) and the colour was read spectrophotometrically at a wave length of 492 nm.

2.4.b. Western blot analysis

Protein concentration was determined by the Lowry assay [26]. Equal amounts (about 20 g/lane) of various protein samples were resolved by SDS-PAGE and electroblotted using a Bio-Rad minigel transfer system (BioRad Laboratories; Cambridge, MA) onto Immobilon P membranes (Millipore; Bedford, MA), using a current of 0.5– 0.75 A for 1 hr. The blots were blocked for 1 hr with 5% non fat dry milk in PBST (0.1% Tween-20) and then incubated with overnight at 4 ^{o}C. MAb at a standard concentration of 2 g/ml was used as primary antibody and further steps were followed as Indirect ELISA. For blot development 3, 3 –diaminobenzidene tetrahydrochloride (Sigma USA) was used.

2.5. SPECIFICITY AND SENSITIVITY OF THE MAB

Six MAbs from the fusion were tested against 12 *L. monocytogenes* standard strains and 69 wild-type isolates obtained from naturally contaminated food products. 10 µg/ml – 20 µg/ml antigen (Whole cell lysate) was coated into 96 well microplate. 50 µl Mabs (1:16000) was added at different concentration into 96 well microtitre plate. The subsequent steps were the same as described in indirect ELISA. To assess the cross-reactivity of the Mab, tests were made using standard strains of *Listeria* along with that closer group of other *Listeria* species and other bacterial genera.

2.6. ANTIBODY ISOTYPING

Subclasses of monoclonal antibodies (immunoglobulins) were identified by isotyping, which is carried out using commercial antibody isotyping kit (ISO2, Sigma, India), followed by manufacturers instructions.

2.6.a. Standardization of simple dot-ELISA

Nitrocellulose paper (NCP) strips of 0.45 m porosity was cut 5 x 5 mm and were coated separately with 5 µl of preparations for the test and *Listeria monocytogenes* bacterial suspension as positive control separately. The negative controls included the NCP coated with preparation from other bacterial speices with coating buffer and incubated in pre-warmed incubator at 60 ^{o}C for 20 mins and blocked in PBS containing 2% BSA or 5% skimmed milk and incubated at 37 ^{o}C for 1 hour. Blocking solution was removed and washed with PBST culture supernatants of hybridomas which has to be tested was added, incubated at room temperature for 30 mins and washed thrice with PBST (1X PBS + 0.05 % Tween), anti-mouse IgG conjugate (1:1000) was added and incubated for 30 mins at room temperature. After washing 5 times with PBST to remove unbound antibodies, the strip was put onto substrate solution consisting of 5 mg of 3, 3-diaminobenzidine tetrahydrochloride (DAB), 10 µl of 38% hydrogen peroxide and 5 ml of 50 mM Tris buffer (pH.7.6) till development of brown coloration. These were then washed in running tap water and dried at room temperature before evaluation.

2.7 Evaluation of standardized dot-ELISA on various field samples

2.7.a. Sample collection:

Food samples (mutton, chicken, fish, pork and ice cream) were collected from local vendors of south India and inoculated into enrichment broth (UVM broth) with selective supplements (Himedia, India). The samples were incubated overnight at 30 ^{o}C in a shaking incubator and a loopful of sample is streaked onto PALCAM agar with selective supplements (Himedia, India) and incubated at 30 ^{o}C for 24 - 48 hrs, suspected grey coloured Colonies with black halo were purified and maintained at 4 ^{o}C until use.

2.7.b. Preparation of whole cell lysate for ELISA:

Overnight cultures of *L. monocytogenes* ATCC 19115 were harvested and washed thrice, with sterile PBS (0.01 M, pH 7.2). The pellet was suspended in sterile distilled water and centrifuged at 10,000 g for 15 minutes at 5 ^{o}C in a Sorvall RC5C centrifuge. Bacterial suspensions were sonicated at 5 watts output for 15 cycles of one minute each with one minute rest in sonicator (Microson). The sonicated material was centrifuged at 10000 Xg for 30 minutes at 5 ^{o}C. The supernatant was filter sterilized using 0.45 um pore size filter and phenyl methyl sulfonyl fluoride (2mM) was added as preservative. The protein contents of the antigens were estimated by Lowry's and stored at -20 ^{o}C until use.

2.8 Molecular detection of *L. monocytogenes* by PCR assay

2.8.a. Preparation of DNA

Bacterial cells pelleted from 2 mL of overnight culture grown in BHI broth was used to prepare genomic DNA by boiling lysis method or by DNA extraction kit (Mackery-nagel). The bacterial cells resuspended in 100 µl TE buffer (10mM Tris and 1mM EDTA) were incubated at 37 ^{o}C for 30 Min along with Lysozyme (10 mg/ml) and then boiled for 15 minutes. The bacterial cell lysates were centrifuged at 12,000 rpm for 2 min and the supernatant stored in -20 ^{o}C for further use.

2.8.b. PCR conditions

PCR amplification was performed in an Eppendorf master thermal cycler (Hamburg, Germany) with the following amplification condition; initial denaturation at 94 ^{o}C for 5 min followed by 30 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 54 ^{o}C for 1 min and extension at 72 ^{o}C for 1.00 min. A final extension of 72 ^{o}C for 8 min was given at the end of every PCR reaction in 20 µl reaction volume. *L. monocytogenes* species specific primer was used. This primer set amplifies a 750 bp product with following primers forward-TGCAAGTCCTAAGACGCAA, reverse-CCACACTTGAGATATATGCAGGA. The PCR products (20 µl) were analyzed by electrophoresis on a 1.0 % agarose gel stained with ethidium bromide

3 Results

3.1 ESTABLISHMENT OF HYBRIDOMA CELL PRODUCING ANTI- *L. monocytogenes* MAB:

Spleenocytes from immunized mice were fused with myeloma cells at ratio 3:1 and hybridomas were selected at HAT medium. Among 200 cultures seeded with fused cells, 180 yielded hybridoma cells (90%) culture supernatants.

The supernatants were removed and screened for antibodies by indirect ELISA. In the primary screening fifteen wells showed reactivity after limiting dilutions, from among 15 wells six clones which were highly reactive and secreted satisfactory amount of antibodies were put to expansion in tissue culture flasks. All ELISA-positive clones thus obtained was maintained at -80 ^{o}C until use.

In order to maximise the chance of isolating a highly reactive antibody, formalin-inactivated *L. monocytogenes* serotype 4b cells were used to immunise test mice. Furthermore, the resultant clones were screened for specificity by plate ELISA (antigen concentration used was 20 µg/ml) and Western blotting. Among 6 clones (Mab107, Mab117, Mab205, Mab103, Mab303, Mab311) which were tested against all the strains, one clone (Mab107) exhibited reactivity with all of the standard strains of *L. monocytogenes* tested, other MAbs exhibited scattered positive results (Table. 2) when tested with other bacterial species.

3.2 WESTERN BLOT RESULTS:
Analysis of the total cellular antigens of *L. monocytogenes* using Western blotting revealed that 1 out of the 6 mAbs were capable of detecting protein band of size 65 kda (Fig. 1), while other failed to react with any protein band on the blots.

Table 2
Specificity testing of Mabs

S. N	Culture	Clone 107	Clone 117	Clone 205	Clone 103	Clone 303c2	Clone 311	PA
1	*Listeria monocytogens ATCC 13932*	+	+	+	+	+	+	+
2	*Listeria monocytogenes NCIM 1143*	+	+	+	+	+	+	+
3	*Listeria monocytogenes NCIM 839*	+	+	+	+	+	+	+
4	*Listeria monocytogenes MTCC*	+	+	+	+	+	+	+
5	*Listeria innocua ATCC 33090*	_	+	+	+	+	+	+
6	*Listeria ivannovi* ATCC 1911	_	+	_	+	+	+	+
7	*Bacillus cereus* ATCC 1457	_	+	_	+	+	_	+
8	*Staphylococcus aureus*	_	+	_	+	+	+	+
9	*Salmonella typhimurium*14 028	_	+	_	+	_	_	+
10	*Shigella boydii* MTCC 1457	_	+	_	+	_	_	+
11	*Escherichia coli* ATCC 9637	_	+	_	+	_	_	+
12	*Vibrio parahaemolyticus* ATCC 17802	_	+	_	_	_	_	+

* PA- Polyclonal antibody

Fig. 1
Western blot with Mab107

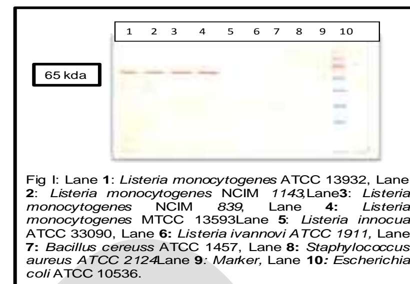
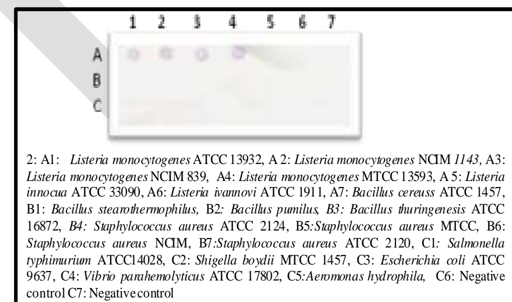


Fig I: Lane **1**: *Listeria monocytogenes* ATCC 13932, Lane **2**: *Listeria monocytogenes* NCIM *1143*,Lane**3**: *Listeria monocytogenes* NCIM *839*, Lane **4:** *Listeria monocytogenes* MTCC 13593Lane **5**: *Listeria innocua* ATCC 33090, Lane **6:** *Listeria ivannovi ATCC 1911*, Lane **7:** *Bacillus cereuss* ATCC 1457, Lane **8:** *Staphylococcus aureus ATCC 2124*Lane **9**: *Marker*, Lane **10**: *Escherichia coli* ATCC 10536.

3.3 SPECIFICITY AND SENSITIVITY
The Mab exhibited a high sensitivity when ELISA was performed. All six mAbs that were shown to react with the WC antigens of *L. monocytogenes* were tested for reactivity with other food-borne pathogens (Table 1) and the non-pathogenic bacterium of the same *Listeria* species *L. innocua, L. ivannovi, L. welshimeri, L. seeligeri, L. grayi* by ELISA. Clone Mab117, Mab103, Mab303, Mab311 reacted with *Listeria* group and also other Gram positive and Gram negative bacteria, clone Mab205 reacted with *L. monocytogenes* and also with other non pathogenic *Listeria* species Mab107 (Fig. 2) reacted with only *L. monocytogenes*.

Fig 2
Dot Elisa Reactivity of clone #107 in Dot- ELISA against confirmed *L.monocytogenes*



2: A1: *Listeria monocytogenes* ATCC 13932, A 2: *Listeria monocytogenes* NCIM *1143*, A3: *Listeria monocytogenes* NCIM 839, A4: *Listeria monocytogenes* MTCC 13593, A 5: *Listeria innocua* ATCC 33090, A6: *Listeria ivannovi* ATCC 1911, A7: *Bacillus cereuss* ATCC 1457, B1: *Bacillus stearothermophilus*, B2: *Bacillus pumilus*, *B3: Bacillus thuringenesis* ATCC 16872, *B4: Staphylococcus aureus* ATCC 2124, B5:*Staphylococcus aureus* MTCC, B6: *Staphylococcus aureus* NCIM, B7:*Staphylococcus aureus* ATCC 2120, C1: *Salmonella typhimurium* ATCC14028, C2: *Shigella boydii* MTCC 1457, C3: *Escherichia coli* ATCC 9637, C4: *Vibrio parahemolyticus* ATCC 17802, C5:*Aeromonas hydrophila*, C6: Negative control C7: Negative control

3.4 PATHOGEN ASSESSMENT OF PURE CULTURES FOR *L. monocytogenes* BY ELISA
Pure cultures from various food samples (Mutton, Chicken, Pork, Fish, Ice cream) which were previously isolated and suspected by conventional method when subjected to newly standardized detection system (dot-ELISA) showed ambiguity. The dot-ELISA results were correlating with PCR results (Table. 3)

Table 3
Comparison of the strains with conventional method, PCR and Dot- ELISA

	No of samples	*L. monocytogenes*		
		API 20	DOT-ELISA	PCR
Mutton	70	28	27	27
Chicken	29	8	8	8
Fish	68	17	16	16
Pork	18	5	6	6
Ice cream	20	5	5	5
soil	18	8	7	7

3.5. ISOTYPING
Out of 6 clones 3 clones (Mab117, Mab205, Mab311) were IgG1 isotype and rest were IgG2A (Mab107, Mab103, Mab303c2)

isotypes.

3.6 MOLECULAR DETECTION OF *L. monocytogenes*

DOT-ELISA results were cross checked with in-house developed PCR method (Fig.3), all the ELISA-positive samples as well as cultures were stayed as positive. Dot-ELISA results equivocally matched with the PCR results.

Fig 3
PCR targeting listeriolysin O gene

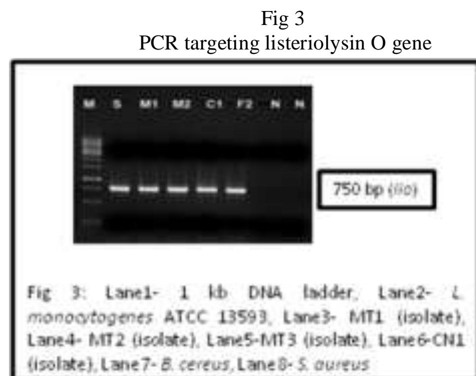


Fig 3: Lane1- 1 kb DNA ladder, Lane2- *L. monocytogenes* ATCC 13593, Lane3- MT1 (isolate), Lane4- MT2 (isolate), Lane5-MT3 (isolate), Lane6-CN1 (isolate), Lane7- *B. cereus*, Lane8- *S. aureus*

4. Discussion

Listeria monocytogenes continues to be an important food borne pathogen worldwide. Although there are rare cases in India, it has proven to cause infections in immunocompromised and pregnant women [7], [10]. Most of the times the cases go under- reporting due to lack of adequate and low cost test system for the proper diagnosis. In the present study, we developed dot-ELISA based method for rapid and specific detection of *L. monocytogenes* from contaminated food and environmental samples. Monoclonal antibodies with specificities for six different epitopes were generated against whole cell antigens from formalinized *L. monocytogenes* cells. Generated hybridoma clones were characterized in terms of specificity and sensitivity using array of bacterial cultures.

Current methods for identification of *Listeria* species rely on physiological and biochemical methods. *Listeria* species are motile by peritrichous flagella and when grown at <30°C, display a characteristic "tumbling" motility. They are catalase positive, oxidase negative and can ferment carbohydrates. On PALCAM agar, all species give grey-green colonies with a black precipitate following incubation for 24 - 48 hours at 35 ^{o}C. This is due to common biochemical characteristics among major listerial species such as esculin hydrolysis, α- Mannosidase production and fermentation of carbohydrates [27]. Therefore, identification of *Listeria* species by conventional detection procedures is cumbersome and lead to ambiguous results.

The binding activities of these mAbs were studied using ELISA technique which is the primary assay to identify affinity of the antibodies towards particular antigen but less efficient in identifying low-affinity antibodies [28] therefore, Western blotting was carried out for all the clones to check cross reactivity with other species of *Listeria- L. ivannovi, L. innocua, L. welshimeri, L. seeligeri L. grayi* as well as with other closely related Gram positive and Gram negative bacteria which is mentioned earlier. Clone Mab117, Mab103, Mab303, Mab311 reacted with *Listeria* group and also other Gram positive and Gram negative bacteria, clone Mab205 reacted with *L. monocytogenes* and also with other non pathogenic *Listeria* species Mab107 reacted with only *L. monocytogenes.* Isotyping results reveals that majority of the mAbs were IgG1 and IgG2a. Out of 6 mAbs obtained 3 Mabs were rapidly detected protein bands at 65 Kda in Western blots of total cellular proteins, other 3 exhibited weak immunological reactions, indicating that the epitopes defined by these antibodies are more immunodominent than other epitopic regions present on the cell surface of the *L. monocytogenes*.

Specific binding to the cell surface with minimal or no cross reaction with other micro- organisms is a desired feature for a mAb to be useful in the detection of *L. monocytogenes*. The lack of cross-reaction of Mab107 with other bacterial species suggests that it has potential for use in the immunocapture of *L. monocytogenes* for rapid detection in food or environmental samples, and for clinical applications such as the detection of the bacterium in infected tissues or cerebrospinal fluid. By using Mab107 a dot ELISA was standardized for which nitrocellulose paper was used which is having high binding capacity for proteins [29] for the rapid and reliable detection of the organism where, after an enrichment step three hours are required to visibly identify the organism by colour development. The technique was applied to identify pure cultures isolated from food samples (mutton, poultry, fish, pork, ice cream) further to check the reliability of developed method PCR assay was standardised targeting 750 bp amplicon of listeriolysin (*llo*) gene for specific detection of *L. monocytogenes.* Listeriolysin is a virulence associated marker and is a secreted pore-forming protein essential for the escape of *L. monocytogenes* from the vacuole formed upon initial internalization [30].

Dot- ELISA results were compared with conventional tests and PCR. Three isolates from mutton, chicken and soil respectively gave confusing results. By biochemical tests these isolates were *Listeria monocytogenes*. When dot- ELISA was performed there was negative reaction for these isolates. To cross check the test a PCR was carried out as mentioned above the dot-ELISA and PCR results correlated with each other therefore, Dot- ELISA can be adopted for detection and it is also proven to be cost effective.

5. Conclusion

The monoclonal antibody based dot- ELISA when evaluated on *Listeria* species and also on other closely related bacteria was found to be robust, specific, and sensitive. Generally routine conventional methods of isolation and characterization are considered as standard method, but are time consuming and less sensitive. This simple dot-ELISA developed in the current study on the other hand can rapidly identify *Listeria monocytogenes.* Therefore, this assay can serve as routine diagnostic tool for identification of the organism.

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